Klinik für Kleintiermedizin
der Vetsuisse-Fakultät Universität Zürich

Direktorin: Prof. Dr. Claudia Reusch

Comparison Of 4 Direct Coombs' Test Methods With Polyclonal Antiglobulins In Anemic And Non-Anemic Dogs For In-Clinic Or Laboratory Use

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Ladina Luzia Caviezel

Tierärztin
von Siat, Graubünden, Schweiz

genehmigt auf Antrag von
Prof. Dr. Urs Giger, Referent

Zürich 2013

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Summary

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Comparison of 4 Direct Coombs' Test Methods with Polyclonal Antiglobulins in Anemic and Non-anemic Dogs for In-Clinic or Laboratory Use

Background: Difficulties with the direct antiglobulin test (DAT) and its apparent lack of sensitivity and specificity for canine immune-mediated hemolytic anemia (IMHA) has raised skepticism regarding its diagnostic value.
Objective: Prospective study comparing different DAT and other methods.
Animals: Anticoagulated blood samples from 59 non-anemic and 46 anemic dogs (+/- IMHA) from a research colony and veterinary clinics.
Methods: Immunochromatographic strip, gel microcolumn and capillary techniques were compared to standard microtiter DAT using 2 polyvalent antiglobulins. Spherocytosis, autoagglutination, osmotic fragility and clinical data were assessed.
Results: Blood samples from all 59 non-anemic dogs were DAT-. Among 46 anemic dogs, 33 were suspected of IMHA, but only 20 were to various degrees DAT+. Spherocytosis and autoagglutination (that did not persist after washing) were noted in 15 and 16 DAT+ dogs, respectively. The other 26 anemic dogs, including 21 previously transfused dogs and 4 with autoagglutination, tested DAT- by different methods. Osmotic fragility was increased in 70% (19/27) of anemic and all 15 DAT+ dogs tested.
Conclusions and Clinical Importance: The novel strip and capillary DAT appear to be promising adjunct in-clinic tools. Despite prior immunosuppressive treatment and presence of autoagglutination, DAT was positive in anemic dogs with IMHA; transfusion did not cause any false DAT+. Our results support DAT as a cornerstone in IMHA diagnosis.

Short Title: Comparison of Various Canine Coombs' Tests.

Key words: Immune-mediated hemolytic anemia; autoantibodies; osmotic fragility; direct antiglobulin test.

Zusammenfassung

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Vergleich von 4 Direkten Coombs-Test-Methoden mit Polyklonalen Antiglobulinen bei anämischen und nicht-anämischen Hunden für den Praxis- oder Laborgebrauch

Hintergrund: Der direkte Antiglobulin-Test (DAT) für die Diagnose der immunbedingten hämolytischen Anämie (IHA) beim Hund wird wegen vermuteter geringer Sensitivität und Spezifität selten klinisch angewandt.
Ziel: Prospektive Studie, die verschiedener DAT Methoden vergleicht.
Tiere: Blutproben von 59 nicht-anämischen, 46 anämischen Hunden (+/- IHA) von Kliniken und einer Hundekolonie.
Methoden: Immunchromatographische Streifen-, Gel- und Kapillar-Techniken wurden mit der DAT-Mikrotiterplatte und 2 polyvalenten Antiglobuline verglichen. Sphärozytose, Autoagglutination, osmotische Fragilität und klinische Daten wurden ausgewertet.
Ergebnisse: Blutproben aller 59 nicht-anämischen Hunde waren DAT-. IMHA wurde bei 33/46 anämischen Hunde vermutet; jedoch waren nur 20 DAT+ mit der Mikrotiterplatte und anderen DAT Methoden. Von den 20 DAT+ Hunden zeigten 15 Sphärozytose, 16 Autoagglutination. Die anderen 26 anämischen Hunde, darunter 21 zuvor transfundierte Hunde und 4 mit Autoagglutination, hatten DAT- Ergebnisse. Alle 15 DAT+ untersuchten Hunde, sowie 70% der anämischen DAT-, wiesen eine erhöhte osmotische Fragilität auf.
Schlussfolgerungen, Klinische Bedeutung: Die neuen Streifen- und kapillare DAT erwiesen sich als vielversprechende Methoden für die Klinik. Anämische Hunde mit IHA zeigten trotz Behandlung mit Immunsuppressiva und Autoagglutination, DAT+ Ergebnisse. Transfusionen verursachten keine falsch+ Resultate. Unsere Studie zeigt die Nützlichkeit des DAT zur IHA-Diagnostik.

Schlüsselwörter: Immunbedingte hämolytische Anämie; Autoantikörper; osmotische Fragilität; Direkter Antiglobulintest.

Abbreviations

AT	antiglobulin testing band
Capillary	capillary tube method
C	control lectin band
DAT	direct antiglobulin test
DEA	dog erythrocyte antigen
EDTA	ethylenediaminetetraacetate
Gel	gel microcolumn method
Hb	hemoglobin
Ig (G, M)	immunoglobulin (G, M)
IMHA	immune-mediated hemolytic anemia
Microtiter	microtiter plate method
min	minutes
n	number
OF(T)	osmotic fragility (test)

Introduction

Dr. Robin Coombs first introduced the antiglobulin test, referred to as the Coombs' test, into human clinical practice in 1945.[1] This immunohematological technique has proven invaluable, specific and sensitive in the detection of erythrocytic auto- and alloantibodies documenting immune-mediated hemolytic anemia (IMHA), hemolytic transfusion reactions and hemolysis of the newborn.[1, 2] The direct antiglobulin test (DAT) detects immunoglobulin (Ig) and/or complement bound to the surface of red blood cells (RBCs).[3, 4] Since the initial conventional tube Coombs' test, several additional methods using microtiter plates, capillary tubes, gel microcolumns, and flow cytometry have been developed. Also, a variety of reagents from polyvalent to specific Ig and complement reagents, under various incubation conditions have been applied.[5]

The DAT has also been used with species-specific reagents in veterinary medicine, mainly in the diagnosis of IMHA in dogs.[6-10] However, difficulties in performing the DAT, subjective result interpretation, and its apparent lack of sensitivity and specificity for IMHA have overshadowed its clinical diagnostic usefulness.[8, 10, 11] Anecdotally, clinicians speak of "Coombs'-negative IMHA" dogs;[7, 12, 13] and some skip the Coombs' test altogether as DAT+ results are rarely received from clinical pathology laboratories. Furthermore, the autoagglutination observed in ethylenediaminetetraacetate (EDTA) tubes or on microscopic slides (with or without adding a drop of saline) is commonly considered sufficient for the diagnosis for IMHA and is also thought to interfere with DAT performance.[9, 14] Moreover, there is a sense that immunosuppressive therapy will immediately convert DAT+ dogs to "false-negative DAT" results and that even one transfusion will cause a "false-positive DAT" result.[14-17] Finally, the erythrocytic osmotic fragility (OF) test (OFT) at specific saline concentrations (5, 50 and 90%) is by some considered a diagnostic test for IMHA.[7, 12]

In light of the perceived uncertainties of the value of the Coombs' test and the introduction of newer techniques, a prospective study aimed at comparing various laboratory and in-clinic

immunohematological DAT methods to detect warm allo- and autoantibodies in anemic and non-anemic dogs prior and/or after treatment and/or transfusion was undertaken. The presence of autoagglutination, spherocytosis and increased OF was also assessed, and clinicopathological and therapeutic information of dogs was evaluated where available.

Materials and Methods

Animals and Samples

From April to October 2012 a free-of-charge extended laboratory assessment of anemic dogs, in particular those suspected of having IMHA, was offered as part of their diagnostic evaluation by notifying ACVIM diplomates and other referral clinicians in the USA via email. Small (1-3 ml) EDTA-anticoagulated blood samples from 24 anemic dogs, freshly collected, chilled and shipped on ice, were received by overnight mail along with similarly prepared samples from 17 healthy non-anemic dogs, to control for shipping artifacts. Leftover EDTA blood samples from 22 anemic and 11 non-anemic dogs submitted to the Clinical Pathology Laboratory at the Veterinary Hospital of the University of Pennsylvania (VHUP) were also assessed, as well as samples from 31 non-anemic dogs in the research colony at the University of Pennsylvania. All samples were stored at 4°C for less than 48 hours until processing. Lipemic samples that could compromise OF testing were not used.

To investigate effects of sample storage, 34 blood samples (11 anemic DAT+ samples and 23 non-anemic DAT- colony dogs) were repeatedly tested by DAT methods on subsequent days after blood collection. Additionally, 9 DAT+ and 8 DAT- anemic dogs were followed up by repeat testing during monitoring of disease progression and treatment response. Signalment (Supplement), routine clinicopathologic and therapeutic information from each dog were also reviewed. Distinction between primary and secondary IMHA was not made. The authors, who established methods and routinely performed these immunohematological techniques, conducted all testing. The Institutional Animal Care and Use Committee at the University of Pennsylvania approved these studies.

Laboratory methods

Routine Hematology Studies –If not already included with the shipped, received samples, blood smears were prepared and stained with Wright Giemsa [a] on arrival for manual microscopic examination. Spherocytosis was recorded, if there were at least 20 spherocytes per 100X microscopic field (all others had <2 per 100X field).[11]

Autoagglutination was assessed first by visual examination of the EDTA tube and by microscopy of the blood smear; if positive, saline agglutination of EDTA blood was performed by adding a drop of physiological saline (0.9% NaCl) to a drop of blood on a slide.[11, 14] Finally, after washing the RBC pellet 3 times by adding 4-6 parts phosphate buffered saline (PBS[b]) to 1 part of packed RBCs, mixing, centrifuging for 5 min at 1000 g[c] and each time discarding the supernatant to remove any remaining plasma proteins; agglutination was evaluated macro- and microscopically. The washed packed RBC samples that retained aggregates were said to exhibit persistent or true agglutination.[9, 10, 18] The PCV of EDTA blood samples was determined by standard microcentrifugation; whole blood and plasma hemoglobin (Hb) concentrations were measured by HemoCue[d]; DEA 1.1 blood type was determined by the immunochromatographic strip method.[19]

To technically validate various antiglobulin tests for this study, conditions for positive and negative reactions were established (Supplement). For this prospective study 3 DAT methods based on RBC agglutination were compared: microtiter[e] plate (Microtiter) using canine antiglobulin reagent M[f] (raised in rabbit) and reagent V[g] (goat), capillary[h] tube (Capillary, Fig. 1A) and gel[i] microcolumn (Gel) technique. Additionally, all samples were tested using a novel immunochromatographic strip[j] method (Strip, Fig. 1B), where antibody coated RBCs migrate and bind to a band impregnated with antiglobulin.

For the various DAT methods, washed RBC suspensions were stored at 4°C for <2 hours, microscopically examined for autoagglutination, and tested according the manufacturers' instructions and as published and briefly described in Supplement. A DAT+ result indicates the presence of

erythrocytic autoantibodies and was used as a criterion for immune-related erythrocyte destruction. A standard OFT was performed, as described in Supplement.

Assessment of various DAT techniques

The various DAT methods applied in the subsequent studies were optimized such that (1) RBCs from DEA 1.1+, but not DEA 1.1- dogs strongly reacted with polyclonal anti-DEA 1.1 alloantibodies, (2) RBCs from DEA 4+ RBCs reacted positively with anti-DEA 4 alloantibodies, and (3) RBCs from 4 known DAT+ dogs with IMHA but not those from 18 healthy control dogs gave DAT+ results with both reagent M and V (Supplement).

Statistical Analysis

Data was entered into a spread sheet[k] and grouped into non-anemic and anemic dogs and based upon diagnostics according to spherocytosis, agglutination, and DAT results. Data was examined for normal distribution. To determine differences between results obtained with various methods, 2x2 tables were used. Kappa (k), the percentage agreement between results of Microtiter-M and other methods beyond chance, was calculated using a clinical research calculator[l]. Based upon OF curves, mean values of hemolysis at OF 5%, 50% and 90% were statistically compared with an independent-sample t-test[m]. Probability values $p<0.05$ were considered statistically significant. Descriptive statistics for the relationships are expressed as mean±SD (PCV, Hb, age, OF).

Results

Direct Coombs' test results in non-anemic dogs

Of 105 dogs studied, blood samples from the 59 non-anemic dogs gave uniformly DAT- results by all methods used. None of these dogs showed any evidence of anemia, hemolysis, agglutination or spherocytosis. (Table 1)

Direct Coombs' test results in anemic dogs

Among the 46 anemic (PCV ≤37%) dogs, 20 were DAT+ and 26 DAT- by Microtiter-M (titer ≥8, used as reference method). Lowest and highest positive titers observed were 8 and 2048, respectively. The failure to agglutinate at low antiserum dilutions caused by a relative excess of antiglobulin in relation to antigen, called the prozone effect, was observed in 4 DAT+ dogs. In 2 dogs, the prozone effect was observed up to a titer of 16 with agglutination seen beyond till a titer of 64 and 128 and in the 2 other dogs the prozone effect was seen till a titer of 64 with final agglutination titer seen at 512 and 1024, respectively.

The degree of DAT positivity (Fig. 2), where gradable, varied but correlated well between techniques: Reagent M gave uniformly equal to twice as strong titers compared to reagent V by Microtiter. (Fig. 2A) All DAT methods used in this comparative study gave similar results when performed at 37°C, 22°C or 4°C, but agglutination with Microtiter was more readily interpretable after incubation at 37°C and 4°C. Among 20 DAT+ dogs determined by Microtiter-M, the Strip revealed weakly positive (1+ to 2+) bands in 12 dogs, strongly positive bands (3+ to 4+) in 7 dogs and missed 1 dog as positive. (Fig. 2B) In contrast, the Capillary and Gel (Fig. 2C) results of the DAT+

dogs gave uniformly strong agglutination reactions, which were easy to read and interpret. Also in the Capillary assay reagent M subjectively gave more and larger beads (agglutination) than reagent V and thus concordant DAT+ results with the following exception: A 4-year-old female spayed Newfoundland with anemia and spherocytosis was only transiently (3 days) weakly to moderately positive by the Microtiter-M (titer 8 and 64) and Capillary test, while all other DAT results were persistently negative. The dog recovered from anemia within 5 days.

The DAT- samples, as determined by Microtiter-M, from 26 anemic dogs were also DAT- by all other methods, with the following 2 exceptions: A 13-year-old castrated male Labrador retriever had an isolated positive Capillary result and was later found to have intestinal leiomyosarcoma but no hemolysis. A 7-year-old spayed female Bernese mountain dog had a weakly positive Strip (1+) result with all other DAT results negative and was later diagnosed with malignant histiocytosis and secondary erythrophagocytosis.

Most notably, results obtained using various DAT methods results were highly concordant: 19 out of 20 DAT+ dogs by Microtiter-M also had DAT+ results with every other method. Agreements between Microtiter-M and V, Gel, Strip or Capillary had kappa values of $\geq$0.94 and a 95% confidence interval of 0.85-1, which represent strong agreements between various DAT results. (Table 2 in Supplement)

Association between clinically suspected IMHA diagnosis, spherocytosis and autoagglutination with DAT results

The PCV and plasma Hb values of DAT+ dogs were significantly lower and insignificantly higher than those of DAT- dogs, respectively, reflecting intravascular hemolytic anemia in some cases. (Table 1)

Among 33 anemic dogs considered by clinicians to likely have IMHA (based on clinical impression, autogglutination on slide and review of blood smear, but rarely performing a Coombs'

test), 13 dogs (39%) were DAT- by Microtiter-M and also by other methods (see 2 singular exceptions above). Further clinical follow-up, when available, revealed that these DAT- dogs had other reasons for anemia, including neoplasia (7), pancreatitis (1), iron deficiency anemia (1), pyometra with surgical complications (1), and cholelithiasis with surgical complications (1). None of these dogs had any evidence of spherocytosis or hemolysis, but 3 of these dogs showed agglutination that did not persist after washing. None of these had any DAT+ results from any outside laboratories.

In addition, 13 of 46 anemic dogs that were not suspected of having IMHA and were DAT- had neoplasia (lymphoma [3], liver mass [2], spindle cell sarcoma [1]), surgical complications (4; lung torsion, cruciate ligament, liver mass surgery, trauma), chronic osteoarthritis (1), hemophagocytic syndrome (1), and pure red cell aplasia (1).

None of the 26 DAT- dogs had spherocytosis, and 4 dogs exhibited agglutination that was not persistent after washing.

All 15 anemic dogs with marked spherocytosis were DAT+ by Microtiter-M and all but one also DAT+ by all other DAT methods; 13 also showed autoagglutination that was not persistent.

Autoagglutination was noted in 20 anemic dogs, but was not persistent and thus DAT could be performed and interpreted: 16 agglutinating samples were DAT+ and 4 were DAT- by all methods. None of the latter had any evidence of IMHA, and were later diagnosed with pure red cell aplasia, malignant histiocytosis, intestinal leiomyosarcoma and multisystemic organ failure, respectively.

Effects of Transfusion on DAT Results

Among 46 anemic dogs, 37 dogs received DEA 1.1-matched RBC transfusions: 11 anemic dogs not suspected of having IMHA were transfused 1 to 21 days prior to Coombs' test performance; all were DAT-. Another 10 dogs clinically suspected of having IMHA, transfused 1 day to 5 years before testing, also had DAT- results; they had neither spherocytosis nor hemolysis. Among 20 DAT+ dogs,

16 were transfused: 12 were tested before receiving a transfusion and 4 had been transfused just 1 to 2 days before testing.

Effects of Immunosuppressive Therapy on DAT Results

Immunosuppressive therapy (glucocorticosteroids and other immunosuppressives) was given to 22 anemic dogs: 12 of 16 DAT+ and 10 DAT- anemic dogs were pretreated with immunosuppressive drugs for 4 days to 2 years (details of dates, drugs, and dosage were not available) before performing DAT; whereas 4 dogs were only immunosuppressed after obtaining DAT+ results.

Follow up DAT Results to Monitor IMHA

Retesting of 8 DAT- and 9 DAT+ anemic dogs for days to weeks revealed that all DAT- dogs persistently remained negative in all DAT analyses. In contrast all 9 DAT+ dogs remained positive for at least 3 days, and 6 were DAT+ for $\geq$10 days. Two dogs followed beyond 10 days became DAT- by 26 and 96 days (Table 3), respectively, and remained DAT-. Concomitantly, the anemia resolved and other hematological parameters normalized. Additionally, 34 DAT- non-anemic research dogs assessed multiple times (6-96 days; average time between tests 40 days) remained DAT-. Finally, samples stored at 4°C from 11 DAT+ dogs tested were consistently positive by all DAT methods for 7 days, while all 23 DAT- samples tested remained negative over the same time span.

Erythrocytic OFT Results

In contrast to 28 non-anemic dogs, which all had normal OF curves, erythrocytic fragility was increased in 70% of 27 anemic dogs tested, including 15/15 DAT+ and 4/12 DAT- dogs (Table 4).

Two of the 4 DAT- dogs with increased OF had lymphoma and in the other 2 the diagnosis remained unknown. All 12 spherocytic DAT+ dogs tested had increased OF with a right shift of the sigmoid OF curve toward physiological saline concentrations. (Fig. 3A) Values of OF 5% and 50% from DAT+ dogs showed a significantly increased degree of lysis compared to healthy non-anemic dogs (Table 4). In the 5 DAT+ dogs that also had spherocytosis and agglutination, the OF curve was not only shifted to the right but also flattened. This specific pattern with slightly decreased lysis around OF 90% and increased lysis at near physiological saline concentration (OF 5%) was not observed in any DAT- anemic dogs. (Fig. 3B)

Discussion

Accelerated destruction of RBCs induced by autoantibodies (and complement) is the hallmark of IMHA.[10, 20, 21] While autoagglutination and spherocytosis may suggest an immune process, only the DAT can specifically document the presence of autoantibodies on RBCs.[2-5, 8] Because of concerns regarding the methodologies, validity, sensitivity, and specificity of the DAT in canine medicine,[6, 8, 9, 11] we compared various DAT techniques, including novel in-clinic tests, using polyclonal canine antiglobulins and other parameters in a select group of anemic and non-anemic dogs prospectively. After establishing and optimizing each technique in our laboratory and confirming that our assays specifically detected erythrocyte bound allo- and autoantibodies, DAT+ results were observed in 20 of 46 anemic dogs, but not in any of the 59 non-anemic dogs. Moreover, observed DAT results correlated between various methods used as well as with the patients' manifestations and other parameters (e.g. spherocytosis) suggestive of IMHA, thereby reestablishing the DAT's clinical usefulness in the diagnosis of IMHA.

Although, there is no DAT gold standard in human and veterinary immunohematology,[8, 16, 21] the tube assay[6] was the standard laboratory DAT technique in the past. However, this is a tedious procedure prone to varied test execution and interpretation of test results.[11] For the studies presented here, we applied a commonly used, but often poorly described, 96-well microtiter plate technique (with reagent M) as a reference method.[2, 11, 13]

In the present study, canine specific polyclonal antiglobulin M (rabbit) was used as reference reagent and results were nearly identical with reagent V (goat) thereby corroborating their specificity. Specific anti-IgG, IgM, and complement reagents were not used to further characterize results of DAT+ samples in this study.

While warm autoantibodies generally cause hemolysis, the best temperature at which to perform the DAT is debatable.[8, 11] The similarity in DAT+ results might be partly due to sequential

performance from 22°C to 37°C and 4°C and extended length of incubation at 4°C. Others[22, 23] also observed that DAT sensitivity could improve after a period of cold incubation: optimal performance was found at both 4°C and 37°C, with both polyvalent and monovalent antiglobulins. Thus, incubation at room temperature may be sufficient to screen for DAT+ anemic dogs, which could simplify and quicken the procedure.

Extended titration of antiglobulin to 2048 not only allowed determination of the strength of the agglutination reaction, but also overcame the previously described interfering prozone effect at lower titers.[8, 11, 16, 23] However, other DAT methods used here were set up with 1 fixed antiglobulin concentration, which, interestingly, gave very similar results and did not seem to affect sensitivity and specificity of DAT results. In a clinical setting, DAT methods with a single reagent concentration could greatly simplify the process and interpretation of results.

The gel microcolumn method uses a standardized procedure to specifically detect antibodies, offers a grading scale for objective interpretation, and has been widely used in immunohematology in humans[2, 24] and more recently in veterinary medicine.[8] This technique became the standard method for canine and feline blood typing.[19, 25] Initial experiences from our laboratory[n] and others[16] with the reagent M containing gel columns used as DAT were very encouraging. Similarly, in the study reported here, the DAT results with the Gel correlated well with the Microtiter-M and all other techniques, but unfortunately the Gel is no longer commercially available for dogs.

The capillary microtube test, introduced in the 1950's[26] to detect antiglobulins, is still being used in human blood banking as a screening test for auto- and alloantibodies.[5] To the authors' knowledge, the study presented here is its first application in veterinary medicine. The Capillary was quick and easy to perform, requiring no special equipment and only small quantities of reagent M (or V), and results were readily interpretable. Moreover, the obtained Capillary results corresponded very well with all other methods used. Thus, the Capillary method may be well suited to become the first in-clinic method to detect the presence of autoantibodies on RBCs in dogs.

The Strip is an innovative and entirely new approach to immunohematology, which recently has already proven invaluable for canine and feline blood typing[19] and is being developed for in-clinic or

laboratory DAT by the same manufacturer. It utilizes an immunochromatographic strip with impregnated reagent M to bind antiglobulin-coated RBCs and thus is not an agglutination-based test. In our experience the Strip was easy to perform, but the resulting band strengths were frequently weak, which could make interpretation a little difficult. Our DAT+ Strip results, which included the weak bands, correlated well with those of other DAT methods and thus can readily be used as an in-clinic screening test.

While various DAT methods results correlated extremely well among the techniques used, DAT+ results also seemed to be restricted to those dogs with evidence of hemolysis and/or suspected of having IMHA. Of the 46 anemic dogs, 33 were suspected by clinicians to have clinically IMHA and 20 were found to be DAT+. Further evaluation of the 13 DAT- anemic dogs did not reveal any specific features of IMHA, and 11 of these DAT- dogs had either evidence of another underlying disorder and/or mechanism of anemia. Thus, the DAT methods used appear to be sensitive and specific to detect dogs with IMHA. The high specificity for all DAT techniques described in this study was similar to that reported in a recent study comparing 2 methods.[11] Most methods, including all used here detecting antiglobulins against RBCs, require previous washing of RBCs, which is a critical technical step, because plasma antibodies not specifically bound to RBCs in an assay, can compete and negatively impact results. In humans with IMHA autoantibodies are not typically removed by repeat washing,[27] they are tightly bound to RBCs and special methods have to be used to elute off the autoantibodies from the red cell surface.[28] However, there is no specific data on autoantibody binding in dogs and any low affinity antibodies being missed by the DAT and causing hemolysis. In the small study presented here there were no cases of suspected IMHA and DAT- results, where there was any other evidence of an immune process causing hemolytic anemia.

Similarly concerns have been raised that the DAT had to be performed immediately following blood collection. However, in this study repeat testing of stored chilled EDTA samples from11 DAT+ dogs gave identical results for one week. Hence, samples prior to emergency or acute treatment can be stored at 4°C and shipped later to a laboratory when deemed necessary to request a DAT without causing false-negative DAT results.

Autoagglutination is frequently considered diagnostic for canine IMHA. In retrospective studies it has been seen in 42-87% of IMHA cases, and is commonly used as a reason to not perform a DAT.[13, 29, 30] However, the degree of agglutination varies, may be unspecific, and may not interfere with Coombs' testing as the agglutination is frequently not persistent. In this study 20 anemic dogs exhibited autoagglutination in tube or on slide, but in all cases agglutination did not persist after washing, which permitted the performance of the DAT. Moreover, the DAT was positive in 80% (16/20) of agglutinating dogs; the other 4 DAT- dogs seemed to have no evidence of hemolysis and had other conditions. Thus, while agglutination may suggest a diagnosis of IMHA, it should be followed up with washing and performance of a DAT to document autoantibody-mediated autoagglutination when possible. Agglutination without washing is shown here to be not diagnostic on its own, with 4 agglutinating dogs having DAT- results; they also had no evidence of spherocytosis and hemolysis and were having other diseases. Beside the data in this prospective study, this has also been observed in many other cases.[10, 18]

Spherocytosis is considered a hallmark feature of IMHA in dogs.[12] In this study severe spherocytosis was seen in 75% of DAT+ and no DAT- dogs. Other studies also observed that 67-94% of dogs affected by IMHA had spherocytosis.[9, 13, 18, 29, 30] However, spherocytes may also be seen with other acquired hemolytic conditions (only few spherocytes)[12] and with hereditary spherocytosis.[31] Moreover, in clinical practice blood smears should be carefully reviewed for spherocytes in the appropriate fields on a slide and preferably analyzed by a veterinary diagnostic laboratory and experienced person or clinical pathologist to avoid false interpretations.[32] Thus, while marked spherocytosis is highly suggestive of IMHA, additional cases are discovered by performing a DAT, which can also confirm the immune mechanism of partial intravascular lysis and/or phagocytosis leading to spherocytosis.[30]

The sensitivity of DAT for IMHA in dogs is widely debated ranging from 50% to 89%.[6, 8, 11, 12, 18, 22, 29, 30] False-negative DAT results have been attributed to technical difficulties with the assay, such as inappropriate or inadequate strength of antisera, exclusive cold agglutinins, failing to adequately wash RBCs, prozone effect, low affinity or quantities of autoantibodies on RBCs or presence of drug-

induced autoantibodies.[6, 11, 14] However, the specific cause has generally not been determined.[8, 14] Optimizing techniques in each laboratory and using standard methods and positive controls could help prevent false-negative DAT results. Based upon analysis of available clinical information in the study reported here, the DAT detected likely all dogs with IMHA. Thus, DAT- dogs should be examined for other causes of anemia. For instance nearly all anemic dogs, found in the authors' laboratory to suffer from hereditary erythrocyte defects, had been assumed to be having IMHA and were previously treated for weeks to years for IMHA and the DAT was either negative or never done (data not shown). The efforts and costs of performing a DAT to confirm a diagnosis of IMHA seem negligible, when considering the costs for other tests, unnecessary treatment and potential side effects of immunosuppressive agents in an anemic dog with other diseases.

Many clinicians believe that administration of immunosuppressive therapy will cause immediately false DAT- results,[9, 14, 16] although the dose and duration of immunosuppressive treatment needed to affect DAT results are poorly documented.[11, 14, 17, 20] This potential drug interference has been used as another reason for not performing a DAT. However, in this study anemic dogs treated with immunosuppression had DAT+ results that remained positive for days to weeks, as previously observed by others.[12, 17] Thus, contrary to common belief, Coombs' testing is recommended in anemic dogs even when already treated. Of course once an animal responds to treatment and goes into remission (normalizes its PCV and has no more evidence of hemolysis), the DAT becomes negative as shown here and elsewhere.[11, 17]

Various reports suggested that previous blood transfusions, especially 1-21 days before performing a DAT, could cause false-positive results.[14, 15] However, no effect of transfusion on DAT could be documented here in this and other studies.[11, 30] This is clinically important, as likely over half of IMHA patients require transfusion during acute management.[13, 29]

The OFT has been developed as a screening test to detect RBC membrane defects such as hereditary spectrin deficiency and stomatocytosis.[31, 33] We also described cats with massively increased OF presumably due to a membrane defect.[34] In light of in the past-perceived difficulties with the DAT, increased erythrocytic OF has been proposed as a diagnostic test result for canine

IMHA. It was assumed that spherocytes or antibody-coated erythrocytes could not withstand lower saline concentrations as well as normal canine RBCs.[12] An increased OF was reported in 85% of dogs previously diagnosed with IMHA.[6] In the prospective study reported here, all DAT+ tested but also some DAT- dogs had increased erythrocytic OF. Thus together with previously reported hereditary and likely other acquired RBC membrane defects resulting in increased RBC fragility, the OFT is not specific for IMHA. Moreover, several DAT+ dogs with autoagglutination and spherocytosis had not only a right-shifted but flattened sigmoid curve: resistant younger RBC population and fragile microspherocytes may explain this unique pattern. However, this complete OFT is fairly cumbersome to perform and may be difficult to interpret particularly if baseline hemolysis exists, thus we cannot recommend this test for the diagnosis of IMHA.

This study included only a small number of animals and cases tested under special laboratory conditions, blood smears were analyzed by clinical pathologists and the person performing DAT was not blinded to other clinical results or other test outcomes. Therefore, further larger studies are needed, with specific protocols, to confirm these promising findings.

In conclusion, this study shows an excellent correlation between various DAT techniques with the reference Microtiter-M method, clinical signs of hemolytic anemia, spherocytosis and true agglutination. As in human medicine, the DAT remains the most sensitive and specific tool to specifically diagnose canine IMHA and seems resilient to storage, immunosuppression and transfusion artifacts.[10, 11, 35] The Capillary and Strip methods are novel and promising simple in-clinic screening tools for IMHA in dogs.

Supplementary Material

Methods

Signalment: Among the 59 non-anemic dogs there were 37 purebred (<3 per breed) and 22 mixed breed dogs, 33 were spayed females and 26 were males; 36 dogs were DEA 1.1+. Of the 20 DAT+ dogs, there were 4 American Cocker Spaniels, 3 Beagles, 3 mix breed dogs, 2 Poodles, 2 Pembroke Welsh Corgis, and one from the following breeds: Old English sheepdog, Jack Russell terrier, Bull terrier, Miniature schnauzer, Newfoundland, and Basenji. There were 12 females (9 were spayed) and 8 castrated males. This group included 12 DEA 1.1+ and 8 DEA 1.1- dogs. The median age at the time of first diagnosis of IMHA was 7 years (range, 1-17 years). Of the 26 anemic DAT- animals, there were 17 purebred (<3 per breed) and 9 mixed breed dogs, 17 were females (13 were spayed) and 9 were castrated males; 16 were DEA 1.1+.

Validation of DAT Techniques: A 5% suspension of 25 µl washed RBCs from each DEA 1.1+ and DEA 1.1- (all were also DEA 4+) non-anemic dogs were incubated at 37°C for 30 min, in subagglutinating concentrations with 25 µl polyclonal anti-DEA 1.1 or anti-DEA 4 alloantibodies (American Blood Resources International), respectively. Thereafter the mixture was washed 3 times with PBS[o] before preparing 5% RBC suspensions for the DAT. Additionally, 4 samples from known DAT+ dogs (not included in study population) were available to optimize the assays prior to the start of this prospective study and to confirm that methods used were detecting bound antibodies on RBCs.

Microtiter-M and -V Test – The DAT in the microtiter plate is similar to the original tube agglutination method, but adapted to a 96-well-plate.[1, 6, 8, 9, 11, 16, 23] Two different commercially available polyclonal canine antiglobulin sera recognizing IgG, IgM and complement were used: Reagent M[p] was raised in the rabbit and reagent V[q] in the goat and the assays were essentially performed according to manufacturers' guidelines and previous studies[6, 16]: Briefly, series of 25 µl

doubling dilutions of antiglobulin (reagent M and V were used in parallel on the same microtiter plate) from 1:2 to 1:2048 were prepared. To each well 25 µl of a 5% RBC suspension were added and thoroughly mixed by use of a semi-automatic micropipette[r]. For each tested sample, one well containing RBCs in PBS (without serum) was used as a negative control to detect autoagglutination. Additionally, a non-anemic control sample was run in parallel with any patient sample as another negative control. Microtiter plates were gently agitated and then incubated initially at room temperature (~22°C) for 30 min. After recording the agglutination reaction of the lowest and highest titers for each sample row, the agglutination was completely dispersed and placed in the incubator at 37°C for another 30 min. After recording those results, suspensions in plates were again swirled gently, covered and placed in the refrigerator at 4°C overnight (12-15 hours). A pellet at the bottom of the well indicates a DAT- result, whereas agglutination, visually revealed by a net of RBCs preventing settling at the bottom, signifies a positive test result. Titer results are expressed as the reciprocal of the highest dilution of antiserum at which autoagglutination is still observed.[16, 36]

Gel Test – The gel in the gel matrix in microcolumn contains reagent M (DiaMed)[s]. Diluent and gel cards were stored at 2-8°C and allowed to warm to room temperature before use. This Gel test was performed according to manufacturer's instructions, using a 1% washed RBC suspension (10µl RBCs in 1 ml low ionic strength saline solution and a specific centrifuge[t] and following guidelines of previous studies[u].[16, 24] Methodologies as well as result interpretation by a grading scale from 0 to 4+ were applied as for blood typing. [19, 25] A grade of ≥2+ agglutination was considered a DAT+ result.

Capillary Test – More than a century ago a capillary tube test was described [37] for the detection of RBC antigens, such as the Rhesus factor in human medicine, using specific antiglobulins. [26] and we adapted this technique for a DAT. A 0.45 mm wide and 7 cm long capillary tube[v] (wider than a typical microhematocrit capillary is dipped into either reagent M or V until one third of the length is filled by capillary force. The antiglobulin-filled end of the capillary is dipped into a 30% washed RBC suspension and an amount roughly equal to the serum is drawn into the capillary. Care must be taken to avoid 'air-locks' between reagent and RBC suspension. The capillary is then inverted, the lower end is sealed and left in clay with an inclining angle of 60°, in order for the RBCs to pass through the

serum for 10 min. [5, 38-40] Positive Capillary test results show beads or clumps of agglutinated cells along the length of the capillary tube, while negative results appear as a thin unbroken red line of cells down the center along the tube wall (FIG.1A); no further grading of degree and or timing of agglutination was attempted.

Strip Test– The principle of this new immunochromatographic strip[w] DAT method, is similar to the DEA 1.1 strips used for blood typing and was performed according to manufacturer's instructions. [19] Briefly, 5 drops of diluent (approximately 225 µl) included in testing kit were placed into one microtiter plate well or a 3 ml glass tube. Washed RBCs (10 µl) were added and mixed for 10 seconds. The tip of the strip was then placed into RBC suspension for 6 min, allowing RBCs to diffuse to the top. The strip is impregnated at 2 levels forming band patterns: a control lectin labeled "C" that binds to all canine RBCs and about 1 cm below is the antiglobulin testing (labeled "AT") site with reagent M. After incubation, the strip was removed and its banding pattern immediately read. A red band at "C" had to be evident for the test to be valid. Any band intensity at "AT" was considered DAT+, indicating the presence of antibodies on the RBC surface. Band strength ranged from 0 (negative), 1+ (positive) to 4+ (strongly positive), based on subjective visual examination (FIG.1B).

Erythrocytic OF Technique– The OFT was performed by adding 15 µl non-lipemic EDTA blood (PCV was adjusted to ~25% by adding PBS or removing plasma) to 2.5 ml of 13 different hypotonic sodium chloride concentrations ranging from 8.5% (physiological saline concentration) to 0% (distilled water), to measure the fragility of RBCs. For every anemic patient assessed, control blood was tested simultaneously. Suspensions were incubated for 45 min at room temperature and then centrifuged for 10 min at 2000 g. The supernatant (300 µL) was pipetted in a 96-flat bottomed well plate and assessed by a spectrophotometer[x] measuring the optical density at 550 nm: the degree of hemolysis at each saline concentration was expressed as percent of the value of complete lysis (sample in distilled water) compared to erythrocytes in 0.85% sodium chloride solution without incubation and OF curves were plotted[12, 34, 41]. Particular attention was given to the specific saline concentrations at which 5% (OF 5%), 50% (OF 50%) and 90% (OF 90%) of erythrocytes were lyzed. One dog was not assessed by OFT because of lipemia.

Results

Assessment of various DAT techniques

In order to assess the various Coombs' test techniques, (1) RBCs from DEA 1.1+ and DEA 1.1- dogs were incubated with polyclonal anti-DEA 1.1 alloantibodies and then DATs were performed. Using the techniques described in the method section, samples from 18 non-anemic DEA 1.1+ dogs gave consistently strongly positive results with the Microtiter, Strip, Gel as well as Capillary; using both reagents M and V. A sample was considered to be DAT+ when the Microtiter was ≥8 and ≥4 with reagent M and V, respectively, or showed 2+ agglutination by Gel, any band at "AT" with the Strip or any beads with the Capillary. In contrast, 15 samples from DEA 1.1- dogs were uniformly negative with all DAT methods applied. Similarly, (2) when using anti-DEA 4 alloantibodies strongly positive results with 4 DEA 4+ dogs were observed, but no DEA 4- samples were available for this study. Finally, (3) RBCs and plasma from 4 known DAT+ dogs with IMHA (initially assessed at VHUP), gave consistently positive results by all methods and both reagents used in this study; while samples from 18 healthy dogs were DAT-.

Conflict of Interest Statement

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Urs Giger has been an unpaid scientific advisor to Alvedia and was not involved in the development of the DAT.

Tables

Table 1

Hematology results of 105 anemic and non-anemic dogs with negative or positive DAT results.

(based upon Microtiter M method)

Anemia	Non-anemic	Anemic	
DAT result	DAT-	DAT-	DAT+
Dogs tested, n	59	26	20
PCV (%), mean ±SD	51.1±4.1	24.4±6.4	17.5±5.6
median (range)	53 (40-56)	25 (11-36)	16 (9-31)
Plasma Hb in g/dl, mean±SD	0.2±0.3	0.3±0.2	0.5±0.8
median (range)	0.0 (0-1.3)	0.2 (0.1-0.5)	0.3 (0-3.3)
Spherocytosis, n (%)	0	0	15 (75)
Autogglutination pre-washing, n/n tested (%)	0/59 (0)	4/26 (15)	16/20 (80)
Increased osmotic fragility, n/n tested (%)	0/28 (0)	4/12 (33)	15/15 (100)

n, number of dogs; SD, standard deviation; Hb, hemoglobin

Table 2

Agreement of DAT results between Microtiter, Gel, Strip and Capillary method as well as spherocytosis, agglutination and osmotic fragility test results, using the Microtiter M method as reference and interrater agreement with Cohen's Kappa value.

Technique	Result	Dogs (n)	True +	True -	False +	False -	Sensitivity (%)	Specificity (%)	Concordance (%)	k	SE	95% CI
DAT+ (strength)												
Microtiter M	≥8	105	20	85	0	0	100.0	100.0	100.0	1	0	1
Microtiter V	≥4	105	19	85	0	1	95.0	100.0	99.0	0.97	0.03	0.91 - 1
Gel	≥2+	99	19	79	0	1	95.0	100.0	99.0	0.97	0.03	0.91 - 1
Strip	≥1+	105	19	84	1	1	95.0	98.8	98.1	0.94	0.04	0.85 - 1
Capillary	+	78	16	61	1	0	100.0	98.4	98.7	0.96	0.04	0.89 - 1
Spherocytosis	>3/10	105	15	85	0	5	75.0	100.0	95.2	0.83	0.07	0.68-0.98
Agglutination	+	105	16	81	4	4	80.0	95.3	92.4	0.75	0.08	0.59-0.92
Osmotic Fragility	Fragile	55	15	36	4	0	100.0	90.0	92.7	0.83	0.08	0.67-0.99

n, Total number of samples tested for each method; +, Positive; -, Negative; k, Cohen's Kappa Value; SE, Standard Error; CI, Confidence Interval

Table 3

Laboratory results of one DAT+ dog with IMHA monitored for 126 days.

Days post treatment	0	7	74	97	126
PCV	17	32	33	38	43
Spherocytes	yes	yes	yes	no	no
Autoagglutination	no	no	no	no	no
DAT	+	+	+	-	-
Microtiter M titer	512	256	256	0	0
Microtiter V titer	128	64	32	0	0
Gel grade	4+	4+	3+	0	0
Strip grade	3+	2+	1+	0	0
Capillary	+	+	+	0	0
Increased Osmotic Fragility	yes	yes	yes	no	no

+, positive; -, negative

Table 4

Osmotic fragility test results: Grouping according to shape of osmotic fragility curves and average saline concentrations for defined lysis values in 55 dogs.

Osmotic Fragility Curves	Anemia	DAT	n	Osmotic Fragility (mean±SD)		
				5%	50%	90%
Normal	no	-	28	0.50 ±0.06*	0.40 ±0.05*	0.29 ±0.10
	yes	-	8	0.54 ±0.05†	0.42 ±0.04†	0.30 ±0.08†
Right shifted	yes	-	4	0.65 ±0.02$^{\dagger\Delta}$	0.52 ±0.01†	0.44 ±0.04$^{\dagger\Delta}$
Right shifted or flattened	yes	+	15	0.76 ±0.07$^{*\Delta}$	0.57 ±0.11*	0.30 ±0.13$^{\Delta}$

n, number of dogs; SD, standard deviation; OF, osmotic fragility

*, difference between mean hemolysis at OF 5% and 50% of 28 non-anemic DAT- dogs with a normal OF and 15 anemic DAT+ dogs with an increased OF ($p=0.002$).

†, difference between mean hemolysis at OF 5%, 50%, 90% of 8 anemic DAT- dogs with a normal OF and 4 anemic DAT- dogs with an increased OF ($p<0.001$).

$^{\Delta}$, difference between mean hemolysis at OF 5% ($p=0.002$) and 90% ($p=0.034$) of 4 anemic DAT- and 15 DAT+ dogs with an increased OF.

Figures

Figure 1

Capillary **(A)** and immunochromatographic strip **(B)** method for direct antiglobulin testing showing positive (**+**) and negative (**-**) DAT results.

Note capillaries are positioned in 60° angle.

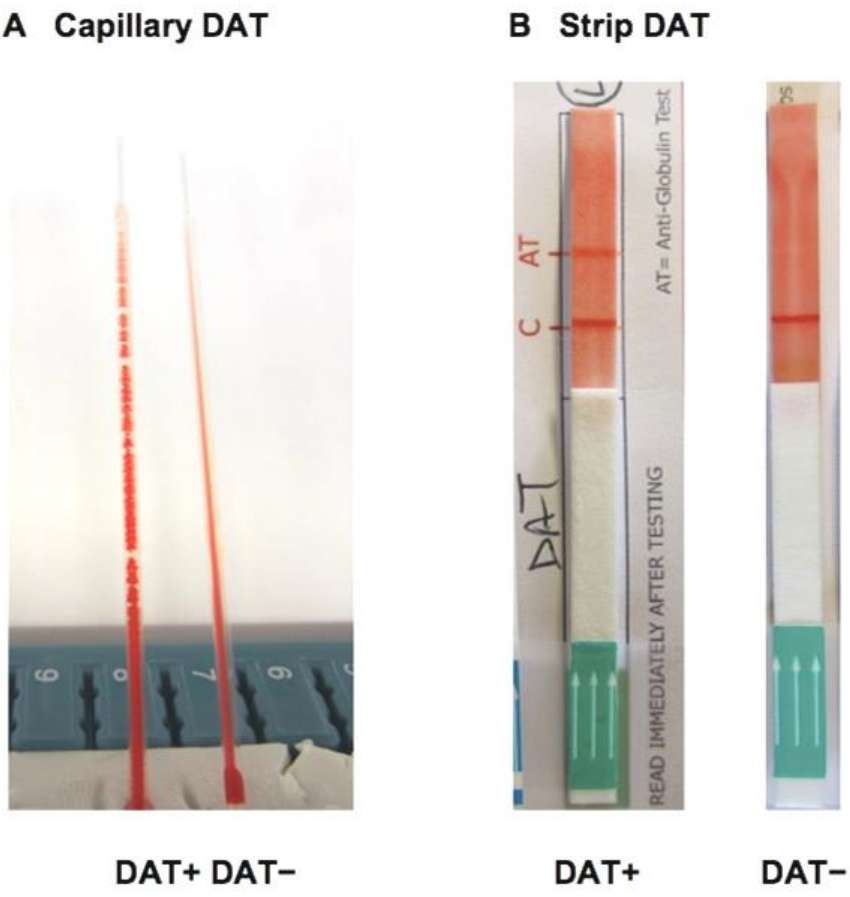
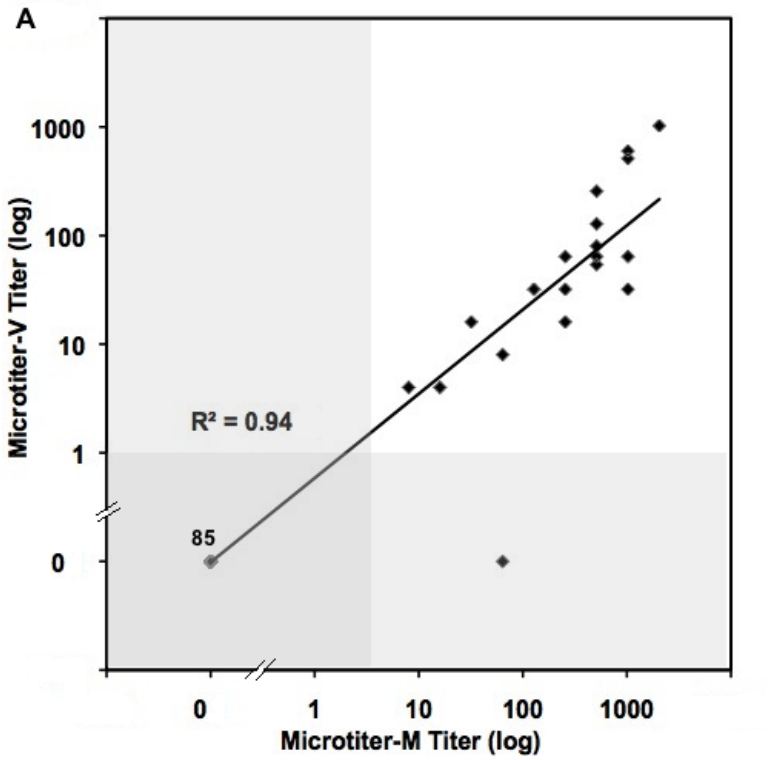
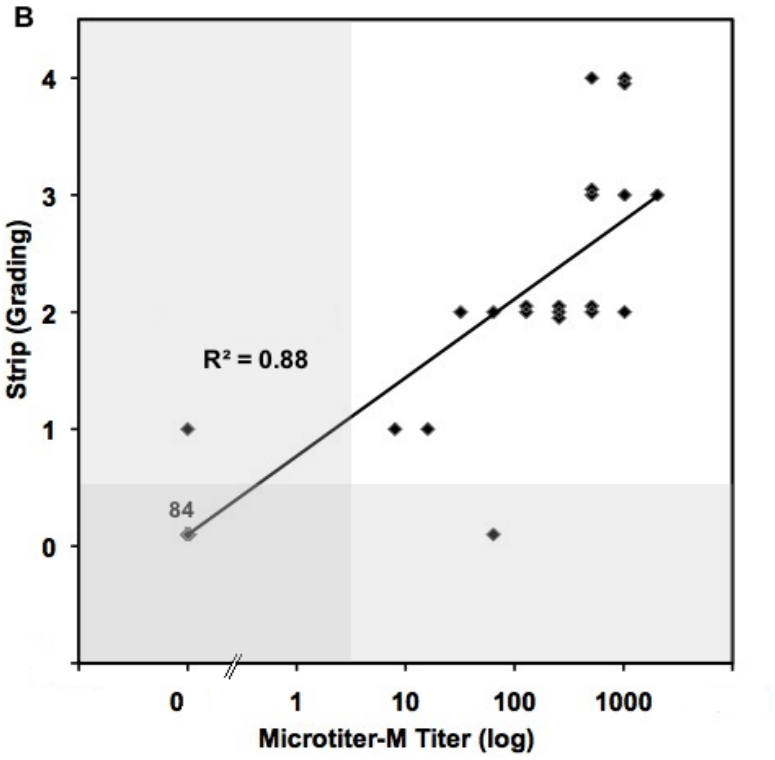


Figure 2

Comparison of DAT results of Microtiter-M with Microtiter-V (A), Strip (B) and Gel (C) methods for 105 dogs. Microtiters are expressed logarithmically; Strip and Gel grading is shown linearly.
Each bullet (◆) represents results from both methods compared for each sample with a linear regression (—). The shaded area refers to the range of DAT- results. Numbers of samples tested are varying, depending on the applied method.




B
Strip (Grading)
4
3
2
1
0
R² = 0.88
84
0
1
10
100
1000
Microtiter-M Titer (log)

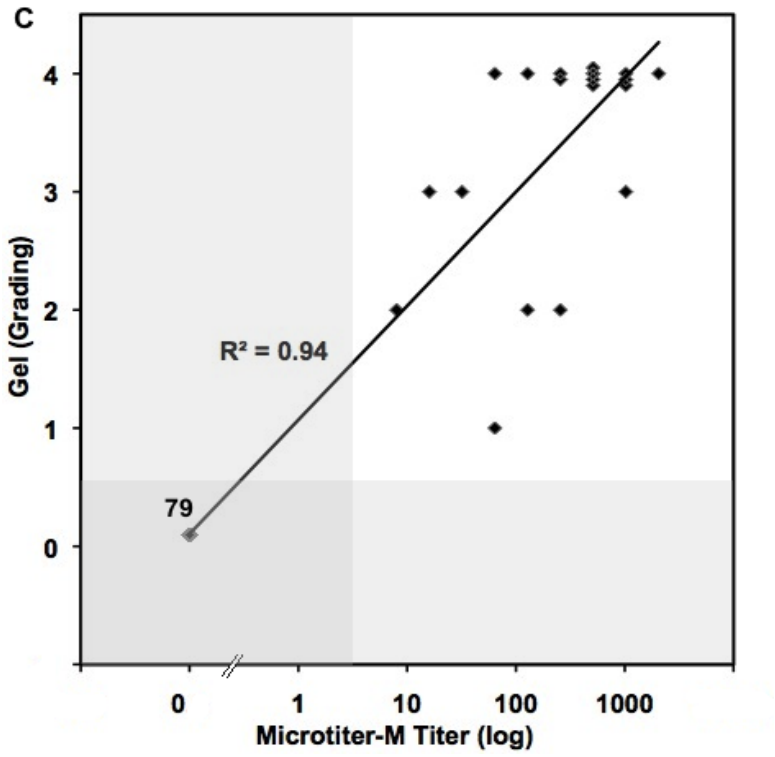
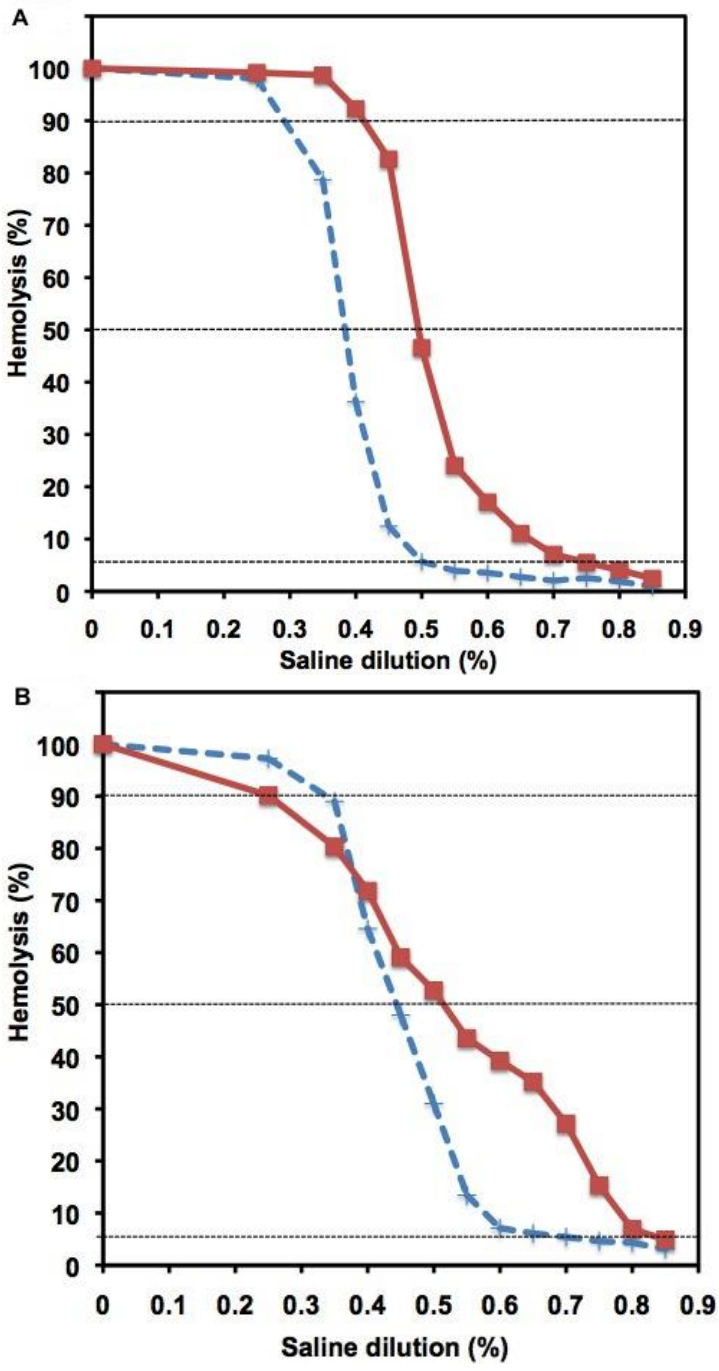

C
Gel (Grading)
4
3
2
1
0
R² = 0.94
79
0
1
10
100
1000
Microtiter-M Titer (log)

Figure 3

Osmotic fragility test curves results curves:

(A) DAT+ ( )and DAT- ( ) control dog. Note right shifted curve for DAT+ dog.

(B) Note right shifted and flattened curve, same as above.



Footnotes

[a] Wright Giemsa stain (Diff Quick Fix, Medion Diagnostics, Düdingen, Switzerland)
[b] DPBS (1x) : Dulbecco's Phosphate Buffered Saline, 1000ml, Gibco by Life Technologies Corporation, Grand Island, NY, USA
[c] Clay Adams Sero-Fuge 2002 Series Centrifuge, BD Diagnostics, NJ, USA
[d] HemoCue® AB- A Quest Diagnostics Company, Ängelholm, Sweden.
[e] 96-well round bottom microtiter plate (Linbro, Flow Laboratories Inc., McLean, VA, USA)
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[m] IBM SPSS Statistics 21, Armonk, NY, USA.
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[u] Jackson KV, Withnall E, Giger U: Initial assessment of a novel gel column Coombs' test to detect auto- and alloantibodies in dogs. Abstract. J Vet Intern Med 21: 623, 2007.
[v] Capillary : Micro-hematocrit capillary tubes- Fischer Scientific, Pittsburgh, PA, USA
[w] Alvedia website. Blood typing quick test and immunochromatographic Antiglobulin Strip, provided by Alvedia, Lyon, France. Available at: http://www.alvedia.com/fr/QT. Accessed April 22, 2012.
[x] Spectrophotometer VersaMax Microplate Reader, Molecular Devices, CA, USA.

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